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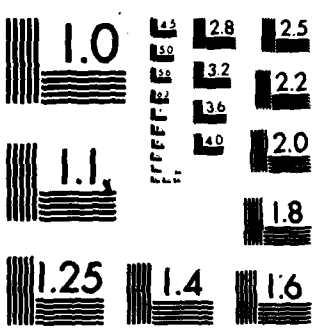

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Annual and Final Report

OUTER MEMBRANE PROTEINS OF PSEUDOMONAS AERUGINOSA: THEIR ROLE IN ANTIBIOTIC SUSCEPTIBILITY

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Experiments were done to measure the uptake of gentamicin (Gen)and dihydrostreptomycin (Sm) by Pseudomonas aeruginosa. Emphasis was placed on the study of: (a) the initial adsorption of Gen and Sm by the outer membrane (OM); and (b) the role played by various ions on their adsorption and uptake. When the Millipore filtration technique was used to measure adsorption and uptake, the amount of initial apparent adsorption that was detected was dependent on how the filters bearing the cells were washed, values being greatest when the cells were washed with water and least when washed with

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solutions of salt.

The apparent amount of antibiotic taken into the cells was also heavily dependent upon the nature of the wash solutions. Equilibrium dialysis experiments showed that Gen and Sm were strongly adsorbed by isolated OM of P. aeruginosa and that ions such as Mg^{2+} prevented this adsorption. The same concentration of Mg^{2+} that increased the MIC of Gen and Sm against P. aeruginosa also prevented their binding to isolated OM. The binding of Gen and Sm to isolated OM appeared to be an ion exchange phenomenon. We concluded from these experimental data that the initial binding of Gen or Sm to the OM is a necessary and essential step in the subsequent uptake of these substances into the cell.

Pseudomonas aeruginosa PAO carrying the resistance plasmid RP1 [R(+) strain] was studied with respect to tetracycline uptake and to the protein profile of the outer membrane (OM). The R(+) strain was compared to the parent strain lacking the plasmid [R(-) strain]. The R(+) strain failed to accumulate tetracycline while the R(-) strain accumulated tetracycline even in the presence of KCN or uncouplers. The absence of tetracycline uptake by the R(+) strain was transiently relieved on exposure to ethylenediaminetetraacetate. The R(+) strain possessed two OM proteins (61,000 and 35,000 daltons) that were not detected in the OM of the R(-) strain; but, a major 45,000-dalton OM protein was detected in the R(-) strain and not detected in the R(+) strain. No differences in the protein profile of the inner membrane of the two strains were noted. The role of the OM protein changes in the R(+) strain is unclear. However, other workers have also detected physical and chemical changes in the OM of P. aeruginosa upon acquisition of this RP1 plasmid. We interpret our experimental data to indicate that the OM acted as a barrier to tetracycline penetration into the R(+) strain of P. aeruginosa.

CONTENTS

LIST OF TABLES

I. SUMMARY

(a) The Role of the Outer Membrane of Pseudomonas aeruginosa in the Uptake of Aminoglycoside Antibiotics.

Experiments were done to measure the uptake of gentamicin (Gen) and dihydrostreptomycin (Sm) by Pseudomonas aeruginosa. Emphasis was placed on the study of: (a) the initial adsorption of Gen and Sm by the outer membrane (OM); and (b) the role played by various ions on their adsorption and uptake. When the Millipore filtration technique was used to measure adsorption and uptake, the amount of initial apparent adsorption that was detected was dependent on how the filters bearing the cells were washed, values being greatest when the cells were washed with water and least when whashed with solutions of salt. The apparent amount of antibiotic taken into the cells was also heavily dependent upon the nature of the wash solutions. Equilibrium dialysis experiments showed that Gen and Sm were strongly adsorbed by isolated OM of P. aeruginosa and that ions such as Mg^{2+} prevented this adsorption. The same concentration of Mg^{2+} prevented this adsorption. The same concentration of Mg^{2+} that increased the MIC of Gen and Sm against P. aeruginosa also prevented their binding to isolated OM. The binding of Gen and Sm to isolated OM appeared to be an ion exchange phenomenon. We concluded from these experimental data that the initial binding of Gen or Sm to the OM is a necessary and essential step in the subsequent uptake of these substances into the cell.

(b) Tetracycline Resistance in Pseudomonas aeruginosa containing the R-Plasmid RP1.

Pseudomonas aeruginosa PAO carrying the resistance plasmid RP1 [R(+) strain] was studied with respect to tetracycline uptake and to the protein profile of the outer membrane (OM). The R(+) strain was compared to the parent strain lacking the plasmid [R(-) strain]. The R(+) strain failed to accumulate tetracycline while the R(-) strain accumulated tetracycline even in the presence of KCN or uncouplers. The absence of tetracycline uptake by the R(+) strain was transiently relieved on exposure to ethylenediaminetetraacetate. The R(+) strain possessed two OM proteins (61,000 and 35,000 daltons) that were not detected in the OM of the R(-) strain: but, a major 45,000-dalton OM protein was detected in the R(-) strain and not detected in the R(+) strain. No differences in the protein profile of the inner membrane of the two strains were noted. The role of the OM protein changes in the R(+) strain is unclear. However, other workers have also detected physical and chemical changes in the OM of P. aeruginosa upon acquisition of this RP1 plasmid. We interpret our experimental data to indicate that the OM acted as a barrier to tetracycline penetration into the R(+) strain of P. aeruginosa.

II. FOREWORD

This contract had an effective date of 1 February 1979 and it terminated 30 June 1980. Thus, the results reported herein represent only seventeen months of research.

III. Background Information

The outer membrane of Gram-negative bacteria constitutes a permeability barrier for hydrophilic substances. This permeability barrier is overcome by outer membrane proteins, called porins, which span the outer membrane and which function as hydrophilic pores mediating the passive diffusion of small molecules through the otherwise impermeable phospholipid bilayer. Nakae (1) and Nikaido (2) have shown that in enteric bacteria the exclusion limit for the passive diffusion of small molecules through porin is 550 to 650 daltons. This exclusion limit was determined by the use of vesicles reconstituted from the individual outer membrane components, protein, lipopolysaccharide and phospholipid. Although porins vary from species to species of Gram-negative bacteria, they generally are major outer membrane proteins of about 30,000 to 40,000 daltons and they exhibit a strong affinity toward peptidoglycan. (For an excellent and recent review of this topic, see ref. 3 by Nikaido and Nakae.)

Many useful antibiotics are hydrophilic and most have molecular weights of about 350 to 500 daltons. Thus, these agents would be expected to diffuse freely through the outer membrane porins of Gram-negative bacteria irrespective whether the Gram-negative bacterial species were enteric bacteria or Pseudomonas. Gram-negative bacteria, however, are intrinsically more resistant to antibiotics than Gram-positive bacteria. According to current concepts, this greater resistance is due to the diffusion barrier posed by the outer membrane (for review, see ref. 4 by Costerton and Cheng and ref. 5 by Brown).

P. aeruginosa is an especially interesting case in this latter regard because, in contrast to findings with other Gram-negative bacteria, Hancock and Nikaido (6) and Hancock et al. (7) reported that P. aeruginosa porin has an exclusion limit of 6,000 ± 3,000 daltons. Thus, with an exclusion limit of 3,000 - 9,000 daltons for P. aeruginosa versus 550-650 daltons for the enteric bacteria, the outer membrane of P. aeruginosa should pose no diffusion barrier to the more common antibiotics. Paradoxically, however, this is not the case because P. aeruginosa is intrinsically more resistant to antibiotics than other Gran-negative bacteria; and, this intrinsic resistance is generally considered to be due to the inability of antibiotics to efficiently penetrate the outer membrane (4,5). It should be noted here that working with intact cells rather than with reconstituted outer membranes, we have evidence that the exclusion limit for the outer membrane of P. aeruginosa is in the same range as for the enteric bacteria (unpublished data). Hancock and co-workers (6,7), in contrast, worked with a reconstituted outer membrane system. Thus, we feel that our intact cell data represent a truer reflection of the actual outer membrane exclusion limits. Moreover, our most recent evidence, which is still highly preliminary, indicates that there is a strain-difference in P. aeruginosa and that the strain used by Hancock and co-workers has wider pores than our strain.

It is now becoming evident that the concept of the porin functioning alone as a hydrophilic pore mediating the passive diffusion of small molecules through an otherwise impermeable phospholipid bilayer must be re-examined. For one thing, pore size (i.e., exclusion limits) has been determined with neutral saccharides and glycols. How porins function with charged molecules as opposed to neutral substances is unknown. Moreover, because a Donnan equilibrium exists across the outer membrane as demonstrated by Roseman and co-workers (8), diffusion of ionized substances is likely to be more complex than simple passive diffusion through porins. Finally, the hydrolytic channel in porin is considered to be negatively charged as shown recently by Benz, Janko and Langer (9).

There are two major types of active transport systems in Gram negative bacteria. One type is cytoplasmic membrane bound and it can be measured in isolated cytoplasmic membrane vesicles. The other type is sensitive to osmotic shock and requires periplasmic binding proteins to support active transport (for review, see ref. 10 by Harold).

The binding proteins are water soluble and they are considered to be located outside the cytoplasmic membrane (i.e., in the periplasmic space). The periplasmic space is external to the cytoplasmic membrane and included in it is the peptidoglycan layer. The outer limit of the periplasmic space, however, is less well defined. In particular, it is not clear to what extent periplasmic proteins may interact with, or be an actual part of, the outer membrane as pointed out by Garrard (11) and Costerton and colleagues (12).

A significant article appeared in 1977 by Boos and co-workers (13) who reported on a "periplasmic" protein related to sn-glycerol-3-phosphate transport in E. coli. These workers concluded that this protein was essential in overcoming the diffusion barrier for sn-glycerol-3-phosphate established by the outer membrane. The location of this protein was not precisely determined, but the evidence suggested that, at least in part, it was outer membrane associated. Moreover, these authors reported that a number of other periplasmic proteins could be surface labeled by lactoperoxidase radioiodination using intact cells. Moreover, Boos and co-workers further reported that porins could also be released by osmotic shock (14).

Other workers have also concluded that specific physical interactions between the "periplasmic" binding protein and the specific cytoplasmic membrane transport component are required for the complete transport process; and, that the binding protein may be also associated with the outer membrane, and most likely in an interaction with porin (for review, see ref. 15 by Lo). Moreover, lipopolysaccharide is also considered to be associated with binding protein as well as with porin; and there is evidence that both free and bound forms of binding protein are associated with different cell envelope components (15). Finally, porin and pore-forming proteins are thought to interact with "free" lipoprotein (14).

In my mind, this evidence now strongly suggests that some "periplasmic" binding proteins may lend substrate specificity to what diffuses through the porin. Thus, the interaction between porin and binding protein would permit the selective diffusion of a specific solute through the outer membrane. The role(s) played by lipopolysaccharide and free lipoprotein in this process is not clear, but these substances may act as "detergents" to keep the strongly hydrophobic outer membrane proteins in proper orientation with respect to the asymmetrical outer membrane.

Minor outer membrane proteins may play a role similar to that of "periplasmic" binding proteins (or, perhaps, what we have been calling periplasmic binding proteins are actually outer membrane proteins); and, there is evidence that some of the minor outer membrane proteins actually do play such a role. One of the most studied is the λ phage receptor which has been implicated in the maltose and maltodextrin transport system by overcoming the diffusion barrier for these sugars through the outer membrane (see Szmelcman and Hofnung, ref. 16). Moreover, Boos and co-workers (17) reported that the λ receptor was able to accomodate small molecules other than maltodextrins. It is significant, however, that the λ receptor is used for maltose transport only when substrate concentration is low. Similarly, minor outer membrane proteins are also involved in the diffusion of other substrates when in low concentration such as vitamin B_{12}, thymidine and sideochrome iron (for review, see ref. 18-20). Thus, by analogy, it is tempting to speculate that minor outer membrane proteins are involved in overcoming the diffusion barrier for anitbiotics through the outer membrane because antibiotics are generally present in low concentration whether in vitro or in vivo. What is clear, however, is that the outer membrane of Gram-negative bacteria has two major types of solute permeation systems: the porin permeability channels; and, as represented by the λ receptor protein, molecule-specific facilitated diffusion channels (21,22). Moreover, there is evidence that the λ receptor protein and the periplasmic maltose-binding protein interact to facilitate diffusion of maltose and maltodextrins (23).

Physiological roles of outer membrane proteins of Gram-negative bacteria other than those just discussed are: peptidoglycan attachment: receptor sites for bacteriophages, bacteriocins, and the F-pilus; and, lipopolysaccharide interaction (3,24,25).

Bacterial resistance to tetracycline is considered to be due to the inability of bacterial cells to take up this substance (for review, see ref. 26 by Chopra and Howe). Most authors consider that the penetration barrier for tetracycline in resistant bacteria is the cytoplasmic membrane. However, expression of resistance is reduced in mutants of E. coli lacking outer membrane protein Ia or containing deep rough lipopolysaccharide, suggesting outer membrane involvement. Moreover genes conferring inducible resistance to tetracycline in the E. coli plasmid R100-1 are contained in the Tn10 transposable sequence which codes for three proteins with molecular weights of 36,000, 25,000 and 13,000 daltons (27). (Other reports have cited molecular weights of 37,000, 27,000 and 15,000 daltons for these proteins [26,38]). The 36,000 dalton protein is an inducible polypeptide and it is referred to as the TET polypeptide. It is considered to be responsible for the decreased uptake of tetracycline in resistant cells of E. coli containing R100 and Tn10 elements. The TET polypeptide is generally considered to be an inner membrane protein (27,28) although some workers consider it to be located either in the periplasmic space or in the outer membrane (26,29). The location of the other two polypeptides is similarly disputed although most authors consider them to be inner membrane components. Regrettably, reconstituted outer membrane vesicles from tetracycline resistant and susceptible cells have not been used to test whether these two vesicle systems have similar or different diffusion properties toward tetracycline.

Bacterial resistance to tetracycline has long been considered to be due to the inability of the bacterial cells to take up this substance. There has not been agreement, however, over whether the outer membrane or the inner membrane is responsible for excluding this antibiotic. Earlier work indicated that the outer membrane of tetracycline resistant cells, and not the inner membrane, excluded the entry of tetracycline into the cells (29,30). More recent work has implicated instead the inner membrane as the exclusion barrier (27,31,32). Most recently, evidence has been presented that tetracycline resistant cells have an active efflux mechanism for tetracycline instead of the inner membrane acting as an exclusion barrier (28,33). On the other hand, tetracycline resistance encoded for by the RP1 palsmid is still thought to be due to exclusion by the outer membrane (34).

Similar to the case of tetracycline, the uptake of aminoglycosides has been measured only by use of intact bacteria. Thus, little concrete information is available on the mechanism of diffusion of these antibiotics through the outer membrane and on the mechanism of their transport through the cytoplasmic membrane. There are conflicting reports on: (a) whether the outer membrane constitutes a penetration barrier (or, if so, what outer membrane component plays a role in the barrier mechanism): (b) whether peptidoglycan constitutes a penetration barrier; (c) whether transport through the cytoplasmic membrane is energy dependent: and (d) whether the cytoplasmic membrane carrier for their transport is inducible or constitutive (for review, see ref. 35 by Nielsen and ref. 36 by Bryan and Van Den Elzen).

Further specific information on the uptake of tetracycline and of the aminoglycosides will be integrated into the following sections of the proposal.

IV. RESEARCH ACCOMPLISHED

(a) Organisms

P. aeruginosa strain NCTC 6750 with and without plasimd RP1, i.e., (R+) and (R-) strains, was obtained from Dr. M.R.W. Brown, the University of Aston in Birmingham, England. According to Dr. Brown, the RP1 plasmid-bearing strains: (a) exhibits resistance to tetracycline, kanamycin, cephaloridine, ampicillin, and carbenicillin: (b) contains periplasmic β-lactamase; (c) is resistant to the lytic action of ethylenediaminetetraacetate (EDTA); (d) is less sensitive to the lytic action of polymyxin B and the lethal action of cold shock: and (e) has a different cell wall composition with respect to the content of cations, of phospholipid and of chemical markers for lipopolysaccharide and peptidoglycan.

P. aeruginosa strain PAO is the most widely studied strain of *P. aeruginosa*. More is known of its physiology and genetics than any other strain. Moreover, its outer membrane (OM) architecture and OM proteins have been characterized not only in my laboratory but in other laboratories as well. For these reasons *P. aeruginosa* PAO(R+) was constructed in my laboratory from *P. aeruginosa* NCTC 6750 (R+) using *E. coli* strain W3110 as the donor. The studies described herein were carried out with *P. aeruginosa* PAO (R+) and (R-) strains.

We also received from Dr. B.E. Ely, the University of South Carolina, *E. coli* strain C600, containing plasmid RP4 Tc^s, and *E. coli* strain J53, containing plasmid RP4.8. (RP1 and RP4 plasmids are now considered to be identical.) These are mutant plasmids with a tetracycline sensitive phenotype and they have been conjugated in my laboratory into *P. aeruginosa* PAO, i.e., strains PAO (RP4 Tc^s) and PAO (RP4.8). These latter PAO strains, however have retained resistance to other antibiotics. The minimal inhibitory concentration (MIC) of the various antibiotics for the various organisms are shown in Table 1.

TABLE 1. Antibiotic Sensitivities of the Various Strains of *P. aeruginosa* and *E. coli*.

Organism	MIC			
	Tetracycline	Kanamycin	Neomycin	Carbenicillin
P. aeruginosa				
PAO (R-)	6	200	25	50
PAO (R+)	400	>800	200	>800
PAO (RP4 Tc^s)	12	>800	200	>800
PAO (RP4.8)	6	>800	200	>800
E. Coli				
W3110 (R-)	<3	<3	<3	12
Wc110 (R+)	200	200	50	>800
RP4 Tc^s	<3	200	50	>800
RP4.8	<3	200	50	>800

In addition to the organisms mentioned above, we have recently acquired P. aeruginosa PAO1, and several auxotrophs of this strain, from Dr. Bruce Holloway from Australia. We have also acquired S. typhimurium LT2 from Dr. Ann Summers, University of Georgia. Finally, we also plan to use P. putida ATCC 12633 in certain studies. Plasmid RP1 has been conjugated into P. putida and P. aeruginosa PAO1. We plan to conjugate this plasmid into S. typhimurium LT2 as well.

(b) Isolation of Outer membranes

Outer membranes were isolated by standard techniques. In one technique, after rupture of the bacteria by the French pressure cell, the cell envelopes were separated by sucrose density gradient centrifugation essentially as has been done by Hancock and co-workers (6,7,37).

The other technique involved the differential extraction of cell membrane from the cell envelope by Triton X-100 as per the technique of Schnaitman and co-workers (38). When the OM proteins from OM prepared by the two techniques were compared, they were identical except for the 1[illegible],000-dalton protein, about 50% of which was lost as a result of the Triton X-100 extraction.

(c) Characterization of OM Proteins in P. aeruginosa Strains

Considerable work on the isolation, characterization and identification of the OM proteins in P. aeruginosa PAO (e.g., peptidoglycan-associated proteins: heat-modifiable proteins; lipoproteins; proteins with porin activity) has been done in the laboratories of Hancock (37) and of Mizuno (39). Therefore, we have been spared this time-consuming basic work and, consequently, we have a source of information with which our OM protein findings can be compared.

Our interest is in what role the various OM proteins might play in the diffusion of tetracycline and the aminoglycosides through the OM. Thus, we have determined what major OM proteins were present in the various strains of P. aeruginosa PAO with which we are working. Our results, as obtained by SDS polyacrylamide gel electrophoresis, are shown in Table 2.

It is apparent that the plasmid RP1/RP4 strains, whether sensitive or resistant to tetracycline, possess two minor OM proteins (57,500 and 33 500 daltons) that were not detected in the plasmid-free strain. Significantly, there was also a 44,000-dalton major OM protein in all tetracycline sensitive strains, whether plasmid-bearing or not, that was not detected in the tetracycline-resistant (R+) strain. Time has not permitted us to determine what role these various proteins might play in the diffusion of tetracycline through reconstituted outer membranes. Likewise, we have not had time to determine whether there is a similar correlation between the OM proteins and sensitivity or resistance to tetracycline in E. coli.

We previously reported that when P. aeruginosa was exposed to EDTA, about 50% of the lipopolysaccharide (LPS) was released as protein-LPS complexes (40,41). These cell free protein-LPS complexes were visualized by electron microscopy (EM) as spherical units 6-7 nm in diameter (40,42); and, via freeze-fracture EM, they were observed in the inner surface of the outer layer of the OM (i.e., concave OM) (42). We also showed that the major OM proteins were components of the protein-LPS complexes (43). Finally, van Alphen and Lugtenberg (44) proposed that these spherical units (i.e., protein-LPS complexes) that we first observed in P. aeruginosa were the porins of the Gram negative bacterial OM.

TABLE 2. OM Proteins in Wild-Type Strain and in Plasmid-Bearing and Mutant Strains of P. aeruginosa

Tentative Nomenclature[a]	Molecular Weight	P. aeruginosa strains			
		PAO(R-)	PAO(R+)	PAO(RP4 Tc^S)	PAO(RP4.8)
	57,500[b]	-	+	+	+
D1	50,500	+	+	+	+
D2	48,000	+	+	+	+
E	45,000	+	+	+	+
	44,000[c]	+	-	+	+
F	35,000	+	+	+	+
	33,500[b]	-	+	+	+
H1	20,000	+	+	+	+
H2	18,000	+	+	+	+
I	9,000	+	+	+	+

[a] Nomenclature proposed by Hancock and Carey (20).

[b] Minor OM proteins detected only in RP1 plasmid-bearing strains. These proteins have not been reported by other workers.

[c] This protein can be resolved from protein E on certain gel systems. Significantly it is absent in strain PAO(R+) which is unable to take up tetracycline while it is present in other strains that are able to take up tetracycline.

Upon re-examination of this system, we confirmed and extended previous observations. Specifically, all of the major OM proteins of P. aeruginosa, including the 44,000-dalton "resistant" protein, were released upon exposure to EDTA (data not shown). We think that the use of the protein-LPS complexes in future work to reconstitute OM vesicles, instead of isolated protein and isolated LPS, may offer certain advantages by maintaining the protein-LPS units in their natural association.

(d) Tetracycline Uptake

When uptake/transport of [^{3}H]-tetracycline was measured by the conventional millipore filtration technique, we found that intact, tetracycline-sensitive cells of P. aeruginosa PAO (R-) took up tetracycline readily; but, tetracycline-resistant PAO (R+) cells did not take up tetracycline. PAO (R+) cells did not require prior cultivation in the presence of tetracycline in order to be fully impermeable to this drug when the external concentration of tetracycline was 1 or 7 µg/ml; but, when an external concentration of 25 µg/ml of tetracycline was used in the uptake studies, prior cultivation of the (R+) cells in the presence of tetracycline enhanced the tetracycline impermeability characteristic indicating some type of inducible system or, instead, perhaps gene amplification, leading to increased amount of "product".

Finally, it is significant that tetracycline-sensitive PAO (RP4 Tc^S) and PAO (RP4.8) cells took up tetracycline at the same rate and to the same extent as the

tetracycline-sensitive, plasmid-less (R-) cells. Thus, these data clearly indicate that the plasmid-borne tetracycline-resistance determinant brings about dramatic changes in the inherent tetracycline uptake system(s) of sensitive P. aeruginosa PAO as has been reported for E. coli by Levy and co-workers (31,32).

When tetracycline-resistant PAO (R+) cells of P. aeruginosa were treated with EDTA, a transient uptake of tetracycline was observed with a rate of uptake identical to that of tetracycline-sensitive (R-) cells. The data suggested that EDTA removed some component(s) from the (R+) cells, most likely from the OM, that acted as a barrier to the diffusion of tetracycline through the OM; and, that upon resynthesis of this component(s), the cells became impermeable once again to tetracycline. This problem is still under study.

Using E. coli R(+) and R(-) cells, we did preliminary experiments to determine whether the cold osmotic technique of Neu and Heppel (45) would have an effect on the uptake of tetracycline by R(-) cells or an effect on the inability of R(+) cells to take up tetracycline. E. coli was used as the test organisms in these experiments because P. aeruginosa is lysed when subjected to cold osmotic shock. Although the results are presently preliminary in nature, it appeared that R(+) cells gained the ability to accumulate tetracycline after cold osmotic shock. The results strengthen the observations mentioned in the preceeding paragraph that incubation of P. aeruginosa R(+) cells with EDTA resulted in the ability of these cells to take up tetracycline. Interestingly, in 1971 Franklin and Foster (29) reported that osmotic shock of tetracycline resistant E. coli resulted in the depression and subsequent recovery of tetracycline resistance. Paradoxically, we found that E. coli R(-) cells lost much of their ability to accumulate tetracycline when subjected to cold, osmotic shock.

When proteins from the shock fluids of E. coli R(+) and R(-) were subjected to polyacrylamide gel electrophoresis, considerable differences in their protein profiles were noted. I hasten to point out that these results presently are highly preliminary and, thus, I will not attempt to draw my conclusions at this time.

Cold osmotic shock of E. coli causes the liberation of periplasmic proteins, of about 50% of the LPS and of a small amount of OM protein (45,46). Which of these components are involved in tetracycline accumulation or exclusion could not be discerned from these preliminary experiments. For that matter the effects may have been at the cytoplasmic membrane level instead. The answer to this question must await further experimentation.

(e) Aminoglycoside Studies

These studies were carried out with gentamicin and streptomycin. Both aminoglycosides appear to be taken up by P. aeruginosa by similar, or perhaps identical, mechanisms. Since we have experienced difficulties from time to time in obtaining [^{3}H]gentamicin, but not [^{3}H]streptomycin, we have used the latter antibiotic more extensively in our studies than the former.

Investigators have long noted that these aminoglycosides are taken up by Gram negative bacteria in three phases (see ref. 47-49 by Davis and co-workers). Bryan and Van Den Elzen (36) proposed that the three phases of accumulation be referred to as: energy-independent (initial) phase; energy-dependent phase I; and energy-dependent phase II. We have been concerned in our work, as described herein, primarily with the first, or energy-independent (initial), phase.

For the past 20 years investigators have noted that there is an immediate adsorption of aminoglycoside, presumably to the bacterial surface, and that much of the aminoglycoside could be removed by washing the cells with salt solutions (49).

Investigators have generally considered this first phase of initial adsorption to be a nuisance; and, in measuring aminoglycoside uptake by the conventional Millipore filtration technique, investigators usually wash the filters bearing the cells with 0.5 M NaCl in order to elute as much adsorbed aminoglycoside as possible (36,49). As a consequence, the nature of the adsorption has received little study. In light of recent understanding of the role of OM proteins (porins) in the diffusion of external solutes through the OM, we concluded that the initial adsorption of aminoglycoside antibiotics onto the bacterial surface was important and worthy of further study.

The initial adsorption of streptomycin and gentamicin appears to be electrostatic in nature. These substances are "bulky", positively charged, water-soluble molecules. They could interact with OM lipopolysaccharide, phospholipids and proteins. Various authors have envisioned the diffusion of the aminoglycosides across the OM as a situation where these drugs first "saturate" the OM and then "promote or mediate their own entry into the cell" (50,51). This is not a very satisfactory explanation.

An increditable amount of aminoglycoside is adsorbed by the Gram-negative bacterial cell within the first 30 sec of incubation. When P. aeruginosa was incubated with streptomycin for 30 sec, then immediately filtered and the filter membranes bearing the cells washed instantaneously with water, streptomycin was concentrated "internally" in the bacterial cells 1,500-fold over the external concentration as calculated on the basis of microbial cell water content (we previously calculated (52) that there are 2.7 µl of cell water per mg dry weight of cells.) When the cells were washed with 0.1 M NaCl and 0.5 M NaCl, streptomycin was concentrated "internally" 960-fold and 160-fold respectively. Very similar results were obtained for gentamicin. These drugs, however, are not considered to be in the physiological interior of the cells; instead, they are considered to be exterior to the cell membrane. Whether they are adsorbed solely into the OM, or whether they are in the periplasmic space as well, could not be discerned by these experiments. We think, however, that the latter most likely is the case.

It has long been noted that divalent cations, such as calcium or magnesium, dramatically increase the minimal inhibitory concentration (MIC) of the aminoglycosides (for review, see ref. 35 and 36). For example, we found that the MIC of gentamicin for P. aeruginosa is 0.78 µg/ml in nutrient broth; 25.0 µg/ml in complex media; and 12.5 µg/ml and 25.0 µg/ml in nutrient broth to which, respectively, 2.5 mM and 7.5 mM magnesium was added in final concentration. Similarly, the MIC of streptomycin was 12.5 µg/ml in nutrient broth, 200.0 µg/ml in nutrient broth containing 5 mM magnesium, and >200 µg/ml in complex media.

Bryan and Van Den Elzen (36) concluded that the diavlent cations prevented accumulation of these drugs into the bacterial cells by acting at the cytoplasmic membrane level. While this may be true, we have evidence that clearly shows that these cations also act at the OM level. These data were obtained by a variety of experimental methods. In one experimental approach, we used the equilibrium dialysis technique with [^{3}H]aminoglycoside in one compartment and isolated OMs in the other compartment (for review of the equilibrium dialysis apparatus and experimental use, see ref. 53 by Furlong and co-workers). As expected, OMs bound the [^{3}H]aminoglycoside and, consequently, all the label appeared in the compartment with the OMs. When magnesium was added to the system, the binding of aminoglycoside was prevented (i.e., equilibrium was reached with equal amounts of aminoglycoside appearing in each compartment). Thus, magnesium clearly prevented the binding of aminoglycoside to isolated OMs. Moreover, there was a strong correlation between the amount of magnesium required to prevent binding of the aminoglycoside to isolated OMs and the amount of magnesium that raised the MIC maximally.

We next attempted to determine the dissociation constant (K_D) for the binding of streptomycin to isolated OMs. When free streptomycin/bound streptomycin was plotted against free streptomycin, the result was not a straight line: instead there were three and possibly four slopes in the plot. These data indicated that streptomycin was being bound by multiple OM components and not just a single component. (If the latter had been the case, a straight line would have resulted.) Thus, a K_D could not be determined in this situation.

We next devised an experiment to determine how much streptomycin was bound "instantaneously" to the OM of P. aeruginosa. This was accomplished by adding a constant quantity of OMs to varying concentrations of aqueous solutions of [^{3}H] streptomycin in centrifuge tubes, mixing and immediately contrifuging to remove the OMs. In this manner we determined how much streptomycin was adsorbed by the OMs after correction for non-specific adsorption onto the centrifuge tube walls. The results showed that approximately 700 nmoles of streptomycin were bound per mg of OM protein. Using this same technique, we determined that 1 μmole of magnesium would prevent the binding of up to about 6 nmoles of streptomycin. Thus, these data show that about a 170-fold higher concentration of magnesium than streptomycin was required to prevent the initial adsorption of streptomycin by isolated OMs of P. aeruginosa.

Magnesium was shown to depress all three phases of streptomycin uptake by P. aeruginosa (i.e., energy-independent initial phase, energy-dependent phase I and energy-dependent phase II). These data were interpreted to indicate that magnesium interferred with the initial adsorption of streptomycin to the OM and thereby prevented ready passage of streptomycin through the OM. Subsequently, the other two pahses of streptomycin uptake were affected by virtue of the fact that the rate of diffusion of streptomycin through the OM was greatly depressed.

It should be noted here that we observed via equilibrium dailysis that isolated cytoplasmic membranes of P. aeruginosa also adsorbed streptomycin and that magnesium prevented this adsorption. This, magnesium possibly acted at the cytoplasmic membrane level as postulated by Bryan and Van Den Elzen (36) as well as at the OM level. However, the inhibition of streptomycin at the OM level by magnesium would be the primary factor in the depressed rate of streptomycin uptake by cells of P. aeruginosa because this drug must first pass through the OM on its way to the interior of the cell.

Holtje (54) concluded recently that interaction of streptomycin with bacterial ribosomes results in the induction of a polyamine transport system which can be utilized for the entry of streptomycin into the cells. When we examined the effect of polyamines on the initial adsorption of streptomycin to isolated OMs, we found that spermine and spermidine were potent competitiors for streptomycin adsorption. Specifically, 40 nmoles of spermine reduced by 80% the binding of 5 nmoles of streptomycin to isolated OMs, while the same concentration of spermidine reduced streptomycin binding by 70%. These results might be expected because the aminoglycosides resemble the aliphatic polyamines, spermine,and spermidine and both classes of compounds show chemical similarities in that they are polycationic molecules containing several unsubstituted imino and amino groups. Thus, while Holtje's conclusions may be correct that streptomycin can utilize a polyamine transport system of the bacterial cell in order to pass through the cytoplasmic membrane, our data also indicate similarities between the adsorption of the polyamines and streptomycin onto the bacterial surface. It is probable, therefore, that both classes of compounds also diffuse through the OM by identical mechanisms.

When the effect of spermine on the uptake of streptomycin by cells of P. aeruginosa was studied, it was found that spermine depressed all three phases of

uptake similarly to that observed with magnesium. The major difference was that spermine was effective at lower concentrations than magnesium. (Needless to say spermine and spermidine also raised the MIC of streptomycin.)

Studies were also done to determine if magnesium, spermine and gentamicin would displace streptomycin initially adsorbed to cells of P. aeruginosa. As might be suspected from the data previously cited, spermine displaced streptomycin more effectively than magnesium; but, gentamicin displaced streptomycin even more effectively than spermine. The reverse situation, however, was not true, namely, streptomycin was only moderately effective in displacing gentamicin. The greater affinity for adsorption noted for gentamicin than for streptomycin is interpreted to be due to the larger number of positive charges on the gentamicin molecule. This may also explain why gentamicin is a more effective antibiotic for P. aeruginosa than streptomycin.

Upon exposure to the aminoglycoside antibiotics, gentamicin or streptomycin. Gram-negative bacteria excrete low molecular weight solutes such as potassium, amino acids and nucleotides (47,55,56). Various authors have concluded that this loss of permeability control is due to mechanical damage, or disorganization, of the cytoplasmic membrane by the drug (for review, see ref. 51 by Franklin and Snow). In order to test this hypothesis, we measured proton extrusion of normal cells of P. aeruginosa and of cells incubated for 30 min in nutrient broth containing 50 µg/ml of streptomycin. We used the technique of Scholes and Mitchell (57) as modified by Rice and Hempfling (58) to measure oxygen-pulsed proton extrusion. Surprisingly streptomycin-incubated cells showed similar proton to oxygen stoichiometry as normal cells. However, the rate of proton resorption by streptomycin-treated cells was greater than that of normal cells. Nevertheless, since streptomycin-treated cells formed an initial proton gradient in a nearly normal fashion, we conclude that streptomycin did not cause mechanical damage to the cytoplasmic membrane.

We also did experiments to determine whether streptomycin had any effect on the ability of isolated cytoplasmic vesicles to carry out active transport. We therefore prepared isolated membrane vesicles from E. coli by the procedure of Kaback (59). It was necessary to use E. coli in this experiment because membrane vesicles prepared from P. aeruginosa by the Kaback procedure are unable to carry out active transport (52). The results showed that incubation of membrane vesicles prepared from normal cells for 30 min in 50 µg/ml of streptomycin had no effect on their ability to actively transport and accumulate proline. On the other hand, when cells of E. coli were incubated for 30 min in 50 µg/ml of streptomycin before they were used as the source of isolated membrane vesicles, then the membrane vesicles isolated from these treated cells showed greatly diminished ability to actively transport and accumulate proline. We conclude, therefore, that the loss of permeability control on exposure of cells to streptomycin requires cellular metabolic activity. Our experiments, however, do not permit us to discern the reason for the loss of permeability control. Nevertheless, our results are highly provocative and, if they bear up after further study, then the question of how and why aminoglycoside-treated bacterial cells excrete intracellular solutes must be reexamined.

We have studied to date only the energy-independent (initial) phase of aminoglycoside uptake. We have not yet studied the energy-dependent phase I and the energy-dependent phase II of the aminoglycoside uptake system. Studies on the latter are planned for the near future. In the meantime, our evidence is highly suggestive that the key to the inhibitory action of the aminoglycosides against P. aeruginosa resides in the composition and structure of the OM.

V. LITERATURE CITED

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VI. PUBLICATIONS SUPPORTED BY THE CONTRACT

(a) Abstracts

(1) The Role of the Outer Membrane of *Pseudomonas aeruginosa* in the Uptake of Aminoglycoside Antibiotics. Saad Abdel-Sayed, Magaly Gonzalez and R.G. Eagon. Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 15 (1980)

(2) Tetracycline Resistance in *Pseudomonas aeruginosa* Containing the R-Plasmid RP1. R.C. Hedstrom and R.G. Eagon. Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 15 (1980)

(b) Full-Length Articles

Three manuscripts are in preparation at this time. When published reprints will be distributed in accordance with pertinent directives.

VII. PERSONNEL RECEIVING CONTRACT SUPPORT

(a) Dr. Robert G. Eagon, Principal Investigator: 1.3 man-months during Summer 1979 and 0.52 man-months during Summer 1980.

(b) Dr. Robert K. Shockley, Postdoctoral Research Associates; 7 man-months for the period December 1979 through June 1980 (i.e., 100% effort for the 7-month period).

(c) Mr. Richard C. Hedstrom, Graduate Student Research Assistant. 4 man-months for the period July 1979 through June 1980 (i.e., 33% effort for the 12-month period).